RELATIONSHIPS BETWEEN SOME GLIAL COMPONENTS OF THE CENTRAL NERVOUS SYSTEM AND THE HAEMOLYMPH-SODIUM CONCENTRATION OF SIX SELECTED INSECTS

By

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CHAPTER I

INTRODUCTION

Several groups of insects, particularly those which have adapted to phytophagous habits, have a haemolymph which does not provide a cation pattern suitable for conduction of nerve impulses by the "sodium mechanisms" which have been found to operate in many other animals (Hodgkin, 1951). Apparently there are special adaptations in the nervous systems of phytophagous insects which allow for the conduction of impulses. Although the adaptations might conceivably be in the physiological mechanism of conduction itself, the studies of Hoyle (1952, 1953), Twarog and Roeder (1956), Yamasaki and Narahashi (1959), Treherne (1961), and Thornton (1963) suggest instead that the adaptations are in the sheaths and glial components which isolate the neurons from the haemolymph. They contend that these sheaths are responsible for providing an ionic environment surrounding the axons different from, and independent of, the haemolymph.

This study is an attempt to define a structural basis for the adaptation of the nervous system of some insects to low sodium haemolymph concentrations.

CHAPTER II

LITERATURE REVIEW

Bone (1944) was the first to analyze the haemolymph of a comprehensive group of insects. His data showed that the haemolymph ionic pattern of insects is quite variable, with phytophagous insects having quite low sodium levels. These observations have since been borne out by analyses by Bone (1946), Tobias (1948a), Duchâteau et al. (1953), Ramsey (1953), Hoyle (1955), and Thornton (1963).

Hodgkin (1951) suggested that a different method of nervous conduction may have evolved in insects because their haemolymph ionic pattern differs considerably from that found in other animals. He suggested that other ions might take the place of sodium and potassium.

Yamasaki and Narahashi (1959) made intracellular measurements of the effects upon the membrane potential of varying the sodium and potassium content of the fluid bathing giant axons in the cockroach. Their results indicate that the "sodium theory" of conduction does indeed apply to this insect.

In addition, the work of Hoyle (1952, 1953), Twarog and Roeder (1956), Thornton (1963), and Treherne (1965a) has shown the necessity of sodium and potassium in insect nerve conduction. The haemolymph of some insects, particularly those with low sodium concentrations, was shown not to have the proper ionic pattern to sustain nervous conduction. Thornton proposed that the glial tissue surrounding the

central nervous system actively transports sodium into the ganglion with the simultaneous exclusion of potassium. In support of this theory, he has shown the concentration of sodium to be higher in the ganglion than in the haemolymph.

Treherne (1961, 1962), after studying the efflux of ^{22}Na ions from the ganglia of the cockroach, whose haemolymph contains a relatively high sodium concentration, suggested that the role of the glial sheaths in concentrating sodium within the ganglion is passive rather than active. He contended that sodium was concentrated in the ganglion by a Gibbs-Donnan equilibrium between extracellular spaces in the ganglion and the haemolymph. However, after studying the ^{22}Na efflux from the stick insect, Carausius morosus, whose haemolymph contains a very low sodium level, he (1965a, 1965b) found that a Gibbs-Donnan equilibrium would not account for all the sodium concentrated in the ganglion. He suggested that the sheath glial cells and possibly other glial cells were responsible for the concentration of the sodium from the haemolymph.

Papers describing the structure of the insect ganglion have been written by Scharrer (1939), Hess (1958), Wigglesworth (1959, 1960), Thornton (1963), and Ashhurst and Richards (1965). The ganglion ultrastructure has been studied by Trijillo-Cenoz (1959, 1962), Ashhurst and Chapman (1961), and Thornton (1963). Smith and Treherne (1963) published a review dealing partially with the histology of the insect ganglion. The ganglion histology will be considered in more detail in a later chapter.

CHAPTER III

MATERIALS AND METHODS

Insects

All specimens studied were collected within fifteen miles of Stillwater. The following insects representing four orders were used:

Order	Scientific Name	Common Name
Orthoptera	Melonoplus differentialis (Thomas)	grasshopper
Coleoptera	Pelidnota lutea L.	grape beetle
Coleoptera	Cicindela cuprascens Lec.	tiger beetle
Hymenoptera	Polistes exclameris Vier	wasp
Hymenoptera	Bombus americanorum (Latreille)	bumble bee
Homoptera	Tibicen priunosa (Say)	cicada

To minimize changes in quantity and composition of the haemolymph due to dehydration, the insects were placed in small vials immediately after collection and stored on ice in a cooler until they could be returned to the laboratory. In most instances, haemolymph samples were taken within three to four hours after the insects were collected, but those insects not sampled immediately were placed in a refrigerator at 10° C.

Analysis of Haemolymph

Two methods were used to sample insect haemolymph. In M. differentialis, P. lutea, T. priunosa and C. cuprascens the haemolymph was collected in capillary tubes inserted through the clypeus. The haemolymph for weighing, drying, and ashing was expelled into crucibles. Because Hymenoptera haemolymph coagulates quickly in capillary tubes, it was collected on small triangular pieces of tared Whatman #42 filter paper inserted into the haemocoel through an opening made by removing the clypeus. After weighing, the filter paper containing the haemolymph was transferred to crucibles for drying and ashing.

The samples were dried to a constant weight at 60° C. in a closed oven and then ashed at 450-550° C. for three to four hours in a muffle furnace. The salts which remained were dissolved in approximately one gram of 0.1 N HCl.

The diluted haemolymph ash was analyzed by flame photometry using a Beckman DU spectrometer fitted with a hydrogen flame attachment (Margoshes, 1962) and compared with known concentrations of sodium.

Histological Procedure

The insects were dissected under saline in waxed bottom petri dishes. The dorsal half of the insect was removed at the level of the spiracles to expose the ventral nerve cord. The nerve cord was cut free with iridectomy scissors and removed with jewelers forceps.

The nerve cords were fixed in Bouin's fixative and left until needed (sometimes several weeks). The nerve cords were dehydrated in 50, 70, 95, and twice in 100% ethyl alcohol, each concentration for

1/2 hour; cleared in two changes of toluene, 1/2 hour each and embedded in tissue-mat (56° C.) (Humason, 1962).

Nerve cords, serially sectioned at 10 microns on a rotary microtome equipped with an Ender's single-edged razor blade, were mounted on slides with Sass' section adhesive (Humason, 1962). Sections were deparaffinized in two, three-minute changes of xylene; hydrated three minutes each in 100, 95, and 70% ethyl alcohol solutions; mordated ten minutes in a solution of saturated mecuric chloride and 5% glacial acetic acid; washed 20 minutes in water; rinsed three minutes each in Lugol's solution, running water, 5% sodium thiosulfate solution and running water. The sections were stained with Mallory's triple connective tissue stain according to Gray (1954). The sections were cleared in xylene and mounted in piccolyte.

Electron micrographs of selected insects were prepared by Dr. John Thornton.

Photomicrography

Light photomicrographs were taken using a Zeiss Winkel microscope with a 35 mm. camera loaded with Kodak Panotomic X film. The negatives were printed on Kodak polycontrast paper using either a number 2, 3, or 4 filter.

CHAPTER IV

RESULTS

Sodium Concentration of Haemolymph

Table I summarizes the results of the sodium analysis. Some of the sample values of the cicada and grasshopper are averages of two separate analyses from the same insect as will be explained later. As would be expected from the work of Bone (1944) and others, the four insects which are phytophagous have the lowest sodium haemolymph concentrations.

A statistical analysis of the data is also given in Table I. The confidence intervals of the insect species are statistically significant at the .05 level as determined by the Student's "t" test (Steele and Torrie, 1960) except for the grape beetle and wasp.

To test the repeatability of the analysis, two samples were taken independently from each of several cicadas and grasshoppers. This was possible because of the comparatively large quantity of haemolymph available from each. This data is given in Table II. In each case the difference between the two observations is not significant even at the .05 level as determined by Student's "t" test (Steele and Torrie, 1960).

The variation observed within a species could be due to the following: (1) differences in age and physiological condition of the specimens, (2) the error inherent in working with small liquid samples,

TABLE I

SODIUM CONCENTRATION OF HAEMOLYMPH

A summary of the flame photometric analysis of the haemolymph of six species of insects for sodium. All values are in terms of mM Na+/1000 grams of haemolymph.

Insect No.	Grape Beetle	Tiger Beetle	Grasshopper	Cicada	Bumble Bee	Wasp
1	88.40	198.43	66.49	28.04	17.17	125.00
2	87.90	205.49	58.11	36.49	17.50	120.00
3	91.60	181.29	86.18	29.22	14.39	105.00
4	99.80	148.75	69.42	35.88	22.34	
5	133.40	219.49	83.82	33.44	29.20	
6	100.80	178.01	72.02	36.84	8.35	
7			63.63	26.57	21.27	
8				31.98		
9				26.85		
$\bar{X}$	100.3	188.6	71.4	31.7	18.6	116.7
Variance	293.4337	617.9394	106.3520	17.4159	43.2530	108.3333
s	17.13	24.86	10.31	4.17	6.58	10.41
$\frac{s}{n}$	6.99	10.15	3.89	1.39	2.48	6.02
$\pm t_{.05} \frac{s}{n}$	17.97	26.10	9.52	3.21	6.07	25.90

TABLE II

ESTIMATE OF PRECISION OF EXPERIMENTAL METHOD

Duplicate samples collected from each of several cicadas and grasshoppers as an estimate of precision. All values are in terms of mM Na+/1000 grams haemolymph.

	Cicada Analysis		Grasshopper Analysis	
Insect	a	b	a	b
1	31.32	24.75	68.56	64.42
2	23.93	34.51	63.43	52.78
3	36.82	34.94	87.99	84.36
4	36.54	30.33	79.27	59.56
5	38.26	35.42	75.45	91.19
6	26.00	27.14	81.06	62.97
7	32.76	31.19	62.16	65.09
8	26.48	27.21		
$\bar{X}$	31.5	30.7	74.1	68.6

$$t_{cicada} = \frac{\bar{D}}{S_{\bar{d}}} = .4337 \text{ N.S.}^{*}$$

$$t_{grasshopper} = \frac{\bar{D}}{S_{\bar{d}}} = 1.2079 \text{ N.S.}^{*}$$

*Not significant at the .05 level

some less than a milligram, and the problem of evaporation before weighing (the magnitude of this error is indicated in Table II which shows the analysis of duplicate samples), and (3) error associated with the flame photometer. When a series of known solutions with values of 100 mM, 75 mM and 50 mM were analyzed, the results were 98.6 mM, 74.6 mM, and 51.3 mM, respectively.

Since the magnitude of error due to (2) above is indicated by the duplicate samples and that due to (3) is small, the remainder of the variation is likely due to actual differences between individuals of the same species. Such individual variations is typically rather large in insects as illustrated by the work of Thornton (1963).

Histological Observations

There are two types of adult insect nervous systems. The abdominal ganglia may be fused with the thoracic ganglia as in the cicada and grape beetle or the abdominal ganglia may persist into the adult stage. If reduced, they are fewer in number than in the larvae stage.

As would be expected from the work of Johansson (1957), Wigglesworth (1959), and Thornton (1963), the histology of the central nervous systems of the six insects studied is very similar. Figures 1 and 2 illustrate the arrangement of the elements.

The entire central nervous system is surrounded by a non-cellular connective tissue sheath. No details are evident with light microscopy, but electron micrographs of the wasp ganglion show a distinctly fiberous construction (Figure 3).

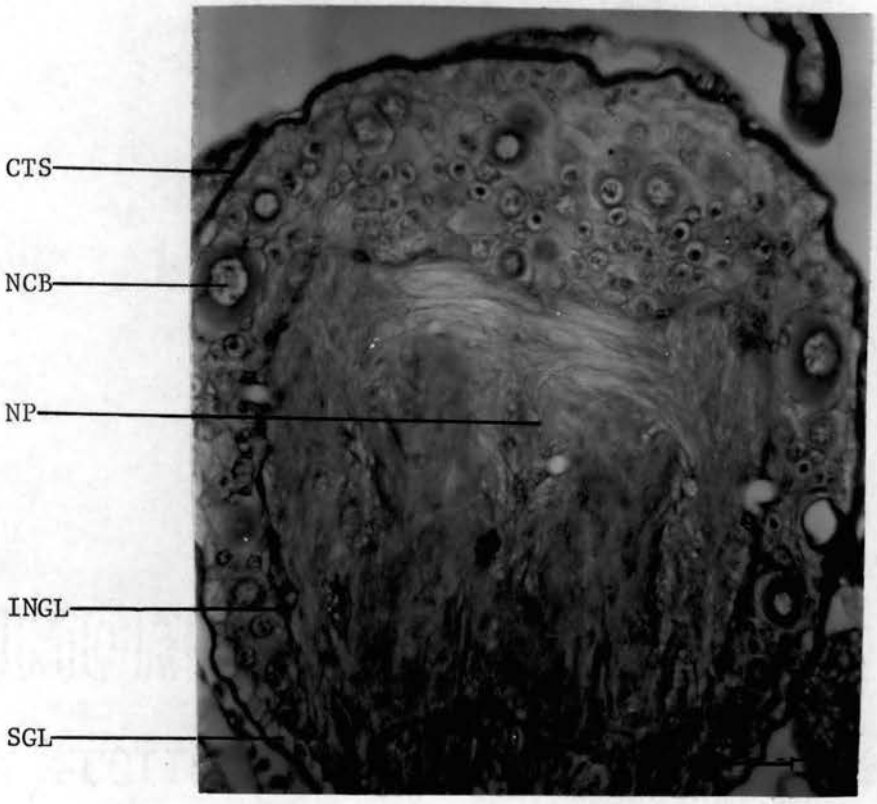

Figure 1. Longitudinal Section Through the Last Abdominal Ganglion of the Grasshopper, Melonophus differentialis. CTS denotes the connective tissue sheath; NCB, nerve cell body; NP, neuropile; INGL, interneuronal glial cells; SGL, sheath glial cells. Scale, 21.8 μ.

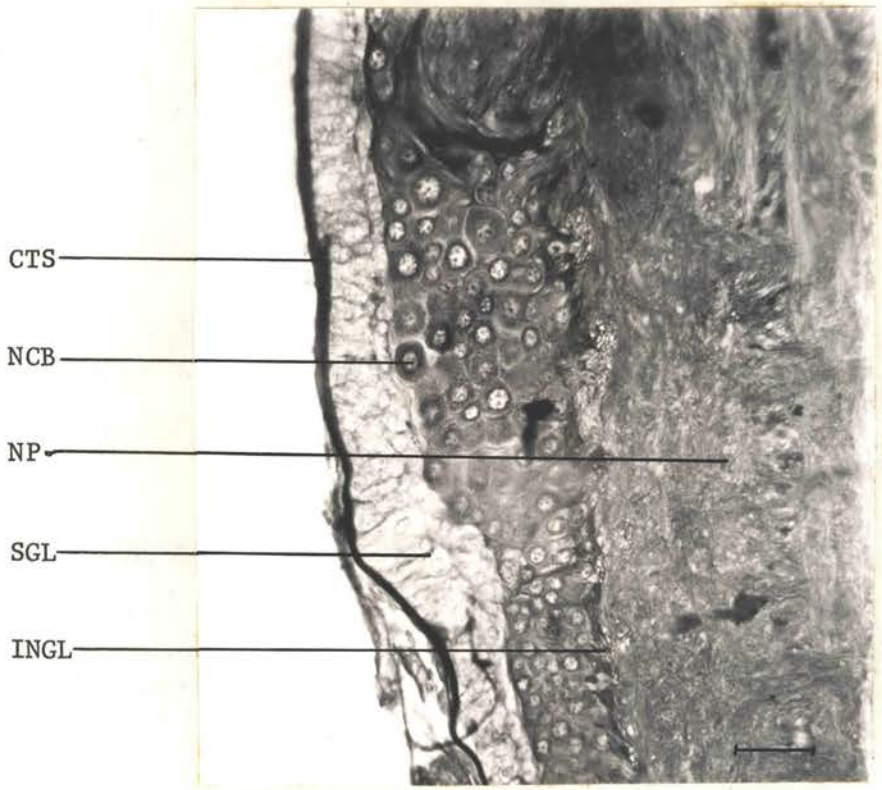

Figure 2. Longitudinal Section Through the Thoracic Ganglion of the Cicada, Tibium priunosa. CTS denotes the connective tissue sheath; NCB, nerve cell body; NP, neuropile; SGL, sheath glial layer; INGL, interneuronal glial cells. Scale, 60 μ.

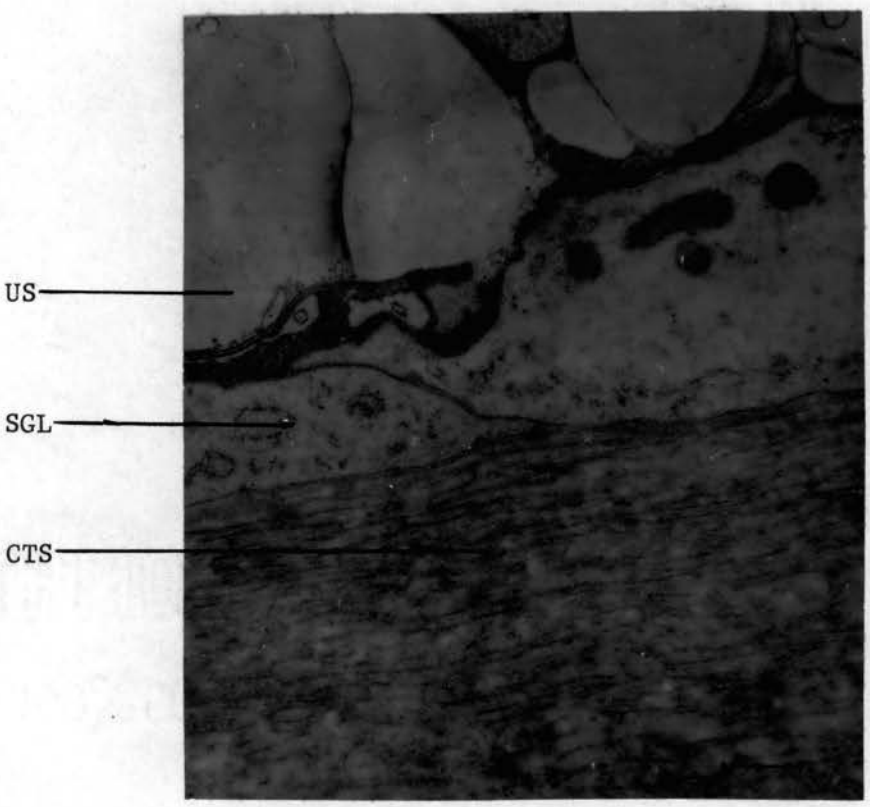

Figure 3. Electron Micrograph of Abdominal Connective of the Wasp Showing the Fiberous Nature of the Connective Tissue Sheath. CTS denotes the connective tissue sheath; SGL, the sheath glial layer; US, unidentified structures. Scale, .63 μ.

Beneath the connective tissue sheath are the sheath glial cells. Electron micrographs show numerous mitochondria in these. This layer, as will be shown later, varies markedly in different species.

The nerve cell bodies with the prominent dark staining Nissl's bodies surrounding the nucleus are located beneath the sheath glial cells. The processes from the nerve cell bodies lead into the neuropile.

Between the layer of nerve cell bodies and the neuropile lies a more or less well-developed layer of glial cells called the interneuronal glial cells. In some insects, as in the cicada, the nuclei of these cells are numerous, large and well developed. In others, as in the tiger beetle, the individual nuclei are very small and infrequent. The cytoplasm for the interneuronal glial cells is not resolved with the light microscope, but electron micrographs clearly show the cytoplasm extending into the neuropile and partially ensheathing the processes there (Figure 4).

The neuropile occupies the central portion of the ganglion. It is here that the synaptic regions of the neurons are located.

If the nerve cell bodies are assumed to be excitable, the only cellular structure lying between the haemolymph and the excitable nervous tissues is the sheath glial layer.

To determine whether a correlation exists between the thickness of the sheath glial layer and the sodium concentration of the haemolymph, measurements of the sheath glial layer were taken using a calibrated ocular micrometer. Three individuals of each species were measured except for the wasp and bumble bee, where only two were measured.

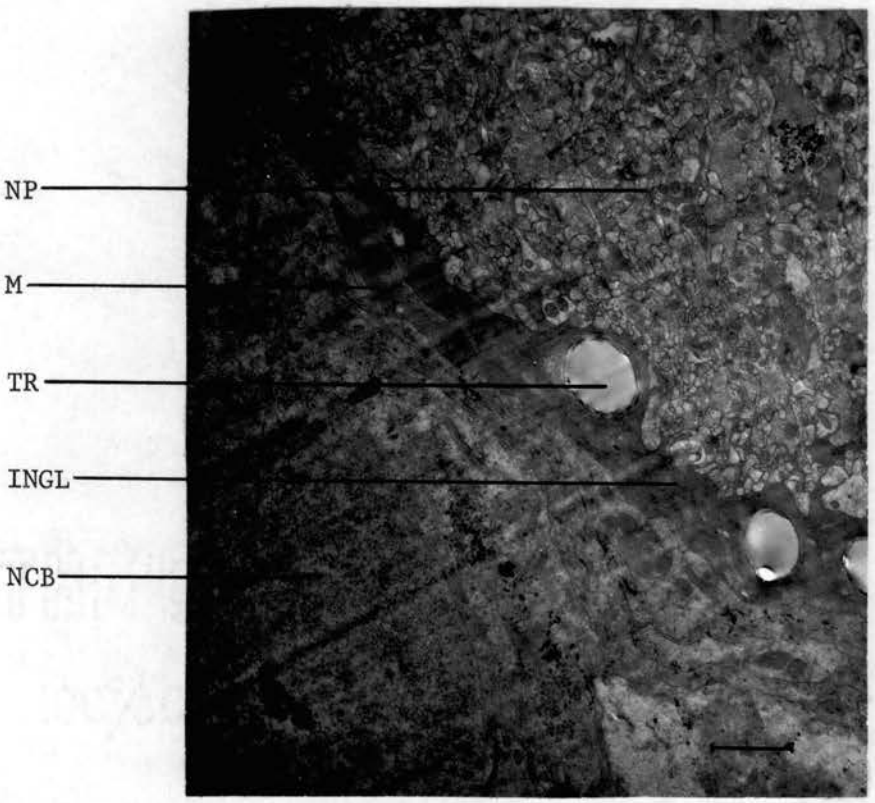

Figure 4. Electron Micrograph of Section From Last Abdominal Ganglion of the Tiger Beetle. Note the numerous large mitochondria along the interneuronal glial layer. NP denotes the neuropile; M, large mitochondria; TR, tracheole; INGL, interneuronal glial layer; NCB, nerve cell body. Scale 1.7μ.

From a serial section of the central nervous system of each individual insect, six sections were randomly chosen. The sheath glial layer thickness was measured at three random positions for each of the six sections selected to be measured. Table III shows the results of these measurements for each insect. The diameter of the ganglion was also measured with the positions to be measured randomly chosen as above.

If the hypothesis that the sheath glial layer concentrates sodium within the extra-cellular spaces of the insect ganglion is true, one would expect that the sheath glial layer would be better developed in those insects with low sodium than in those with high sodium. This development might be reflected in their size. Table IV shows that there is a correlation of -.46 between the thickness of the sheath glial layer and the haemolymph sodium concentration in the insects studied.

To compensate for the differences in ganglion sizes between the species being studied, the correlation between a correction ratio and the haemolymph sodium concentration was calculated. The correction ratio was computed by dividing the ganglion thickness into the sheath glial layer thickness. The correlation shown in Table V is -.47. For the insects under consideration, the correlation compensating for the ganglion size differences appears to be the same as the correlation computed without regard to size.

Another function for the sheath glial cells as has been postulated by Ashhurst (1959, 1961, 1964) is that of secreting the connective tissue sheath. In support of this, a positive correlation of .63 was

TABLE III

SHEATH GLIAL DATA ANALYSIS

The sheath glial cell layer of the six following insects was measured. The statistical analysis of the data is given below. All units of measurement are in microns.

Insect	$\bar{X}$	$\frac{s}{n}$	$\pm t_{.05} \frac{s}{n}$	C. I. Limits*
Tiger beetle	2.58	.2524	1.09	1.49-- 4.67
Grape beetle	21.10	2.325	10.00	11.10--31.10
Cicada	64.77	68.51	29.48	34.29--94.25
Bumble bee	4.12	.3123	3.97	.05-- 8.09
Grasshopper	10.24	.3066	1.32	8.92--11.56
Wasp	3.78	.9400	11.94	0.00--15.72

*At the 95% level

TABLE IV

CORRELATION BETWEEN SHEATH GLIAL THICKNESS AND SODIUM CONCENTRATION OF HAEMOLYMPH

The correlation between the thickness of the sheath glial cell layer and the sodium concentration of the haemolymph was calculated. x is thickness of the sheath glial cell layer in microns, y is sodium concentration of the haemolymph in mMoles/1000 grams haemolymph, and r is the correlation between x and y.

Insect	x	y
Tiger beetle	2.58	188.6
Grape beetle	21.10	100.3
Cicada	64.77	31.7
Bumble bee	4.12	18.6
Grasshopper	10.24	71.4
Wasp	3.78	116.7

$$r = \frac{\Sigma xy}{\sqrt{\Sigma x^2 \cdot \Sigma y^2}} = -.46$$

TABLE V

CORRELATION BETWEEN THE GANGLION SIZE CORRECTION RATIO AND THE SODIUM CONCENTRATION OF THE HAEMOLYMPH

To compensate for the differences in ganglion size in different species, the correlation between a correction ratio (glial sheath thickness/ganglion diameter) and the sodium concentration of the haemolymph was calculated. x is the correction ratio, y is the sodium concentration in the blood in mMoles/1000 grams, and r is the correlation between x and y.

Insect	x	y
Tiger beetle	.0083	188.6
Grape beetle	.0352	100.3
Cicada	.0926	31.7
Bumble bee	.0101	18.6
Grasshopper	.0199	71.4
Wasp	.0116	116.7

$$r = \frac{\Sigma xy}{\sqrt{\Sigma x^2 \cdot \Sigma y^2}} = -.47$$

calculated, as shown in Table VI, between the thickness of the sheath glial layer and the thickness of the connective tissue sheath.

More difficult to understand, however, is the correlation of -.79 between the thickness of the connective tissue sheath and the sodium concentration of the haemolymph shown in Table VII. Since this sheath in the insects under study is acellular and has been shown by Wigglesworth (1960) and Twarog and Roeder (1956) to be readily permeable to large ions, it is doubtful that it plays any role in the regulation of ions in the ganglion. Apparently, support is its primary function. This will be discussed more fully later.

TABLE VI

CORRELATION BETWEEN SHEATH GLIAL THICKNESS AND CONNECTIVE TISSUE SHEATH THICKNESS

The correlation between the thickness of the sheath glial cell layer and the connective tissue sheath was calculated. x is the thickness of the sheath glial cells in microns, y is the thickness of the connective tissue sheath in microns, and r is the correlation between x and y.

Insect	x	y
Tiger beetle	2.58	0.65
Grape beetle	21.10	4.5
Cicada	64.77	5.5
Grasshopper	10.24	3.3
Wasp	3.78	1.5
Bumble bee	4.12	3.0
$r = \frac{\Sigma xy}{\sqrt{\Sigma x^2 \cdot \Sigma y^2}}$	$r = +.63$	

TABLE VII

CORRELATION OF CONNECTIVE TISSUE SHEATH TO SODIUM CONCENTRATION

The correlation between the connective tissue sheath thickness and the sodium concentration of the haemolymph is shown below. x is the sodium concentration in the haemolymph in mMoles/1000 grams of haemolymph, y is the connective tissue sheath thickness in microns, and r is the correlation between x and y.

Insect	x	y
Tiger beetle	188.6	0.65
Grape Beetle	100.3	4.5
Cicada	31.7	5.5
Grasshopper	71.4	3.3
Wasp	116.7	1.5
Bumble bee	18.6	3.0
	$\bar{X}$ = 89.0	$\bar{Y}$ = 3.1
$r = \frac{\Sigma xy}{\sqrt{\Sigma x^2 - \Sigma y^2}}$		r = -.79

CHAPTER V

DISCUSSION

If the Hodgkin-Huxley model of nerve conduction applies to the primitive condition in Arthropods, an insect having a food source limited to materials with a low concentration of sodium, must evolve one or more of the following three adaptations: (1) a mechanism to concentrate sodium in its haemolymph, (2) a mechanism to concentrate sodium around the neuronal tissues, or (3) a mode of nerve conduction not dependent upon high extracellular sodium concentration. Electrophysiological data (Hoyle 1952, 1953; Twarog and Roeder 1956; Thornton 1963; and Treherne 1965a) indicate that many insects have evolved the second alternative.

Because the nerve cell bodies of some molluscs have been shown to be nonconductive, there is some question as to whether or not insect nerve cell bodies are excitable. If it is assumed that they are, the only visible cellular tissue lying between the conductive tissue and the haemolymph, with the exception of glial tissue among the nerve cell bodies, is the sheath glial cell layer. It has been postulated by Thornton (1963) and others that these cells regulate the cation pattern within the ganglion by concentrating sodium in the interstitual spaces of the ganglion in those insects that have low sodium levels in the haemolymph. If this is true, the degree to which sodium is transported into the ganglion should be reflected in the development of the

sheath glial cells. Indeed, this study has shown a correlation of -.46 between the level of sodium in the haemolymph and the thickness of the sheath glial layer.

Ashhurst (1959, 1961, 1964) has postulated that the connective tissue sheath is laid down by the sheath glial layer. In support of this theory a correlation of +.63 was found between the thickness of the sheath glial cells and the connective tissue sheath. The correlation jumps to +.81 when the works of Wigglesworth (1959) on Rhodnius and Thornton (1963) on the sphinx moth are considered. Although the connective tissue sheath might conceivably be produced by cells in the haemolymph, at this time the evidence points toward their formation by the sheath glial cells.

A correlation of -.79 was found between the connective tissue sheath thickness and the level of sodium concentration in the haemolymph. This is unexpected since Wigglesworth (1960) and others have ruled out the possibility that the acellular connective tissue sheath has a direct effect on the regulation of ions in the ganglion. That this correlation is so much higher than that found for the sheath glial cells is even more surprising. A possible explanation is that the acellular connective tissue serves as an ion binding agent, thus increasing the total sodium available for transport by sheath cells. There is, however, no direct evidence to support this.

If the nerve cell bodies should prove to be nonconductive, there are other glial cells, in addition to the sheath glial cell layer, which lie between the haemolymph and the conductive tissue. The space between the nerve cell bodies is filled with glial cells and there is a well defined layer of glial tissue between the nerve cell bodies and

the neuropile. The structure of this layer is not well resolved by light microscope study, but electron micrographs show large numerous mitochondria in the layer, suggesting an important metabolic function. To date no work has been done to try to determine the exact function of this layer.

There are two major problems which confound a study of this sort: (1) The sheath glial cells are definitely involved in other functions such as support -- laying down the connective tissue sheath -- and nutrition (Wigglesworth, 1960). Their interfering effect on the size of the sheath glial layer thickness is unknown at this time. (2) An insect might change sources of food several times during the course of their evolution. The type of food, whether vegetative or animal, most plentiful and easily obtainable, is undoubtedly a strong evolutionary force shaping the food habits of a particular insect. Morphological and, or physiological adaptations needed because of the ionic composition of a particular diet might be partially or completely retained even though not needed if the insect changed to foods having a different ionic make up from the preceding diet.

CHAPTER VI

SUMMARY

This study was initiated in an attempt to determine which structural elements in the central nervous system contributed to the adaptation of the nervous system of some insects to low sodium haemolymph. The following is a summary of the data obtained.

1. The sodium analyses established that the haemolymph sodium concentrations of the six insects studied followed the general pattern of low sodium concentration for phytophagous insects and high sodium concentration for carnivorous insects. The difference in haemolymph sodium concentration between the two types of insects may not be greatly marked, however.

2. A correlation of -.46 was found between the thickness of the sheath glial cell layer and the sodium concentration of the haemolymph. In the insects under study correction for differences in ganglion size did not change the correlation.

3. A positive correlation of .63 exists between the thickness of the sheath glial layer and the acellular connective tissue sheath, adding evidence to the theory that the sheath glial layer produces the connective tissue sheath.

4. A negative correlation of -.79 was found between the thickness of the connective tissue sheath and the sodium concentration of the haemolymph.

5. The layer of interneuronal glial cells lying between the nerve cell bodies and the neuropile was markedly better developed in some insects than in others. The reason for this is unknown at this time.

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